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Luteolin induces caspase-dependent apoptosis *via* inhibiting the AKT/osteopontin pathway in human hepatocellular carcinoma SK-Hep-1 cells

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ABSTRACT

Aims: Luteolin, a naturally occurring flavonoid, possesses anti-cancer effects including induction of apoptosis. This study investigated the involvement of osteopontin (OPN) in luteolin-induced apoptosis in human hepatocellular carcinoma (HCC) SK-Hep-1 cells with high OPN expression.

Main methods: MTT assay was used to determine the cell viability. Cell cycle analysis was performed to identify apoptosis. Apoptosis was confirmed by detecting cytoplasmic histone-associated-DNA-fragments using a cell death detection ELISA^{PLUS} kit. The expression of proteins was evaluated by Western blot. Reverse transcriptase-polymerase chain reaction was employed to detect the expression of mRNA.

Key findings: Cytotoxic effect of luteolin was higher in cancer cell line SK-Hep-1 cells than in normal cell line AML12 cells. Luteolin led a significantly increase in apoptosis accompanied by activation of caspase 8, −9 and −3 and cleavage of poly (ADP-ribose) polymerase (PARP), which was completely blocked by *Z*-VAD-FMK, a pan caspase inhibitor. Luteolin significantly downregulated the expression of X-linked inhibitor of apoptosis (XIAP), Mcl-1 and Bid. Furthermore, luteolin effectively decreased OPN expression at both mRNA and protein level. Exogenous OPN markedly blocked apoptosis induction, caspases activation, PARP cleavage, downregulation of XIAP and Mcl-1 in luteolin-treated cells. Luteolin impaired the AKT pathway by inhibiting the phosphorylation of AKT. SC79, an AKT activator, blocked apoptosis induction, caspases activation, PARP cleavage, downregulation of OPN, XIAP, Mcl-1 and Bid in luteolin-treated cells.

Significance: These results demonstrated that luteolin inhibits the AKT/OPN pathway, thereby inducing caspase-dependent apoptosis in human HCC SK-Hep-1 cells with little toxicity.

1. Introduction

Flavonoids found in fruits, vegetables and medicinal herbs have beneficial effects to human and animal health through a broad spectrum of pharmacological activity including anti-cancer effects [1–3]. Luteolin, a naturally occurring flavonoid, has been used traditionally for hypertension, inflammatory diseases, and cancer in Chinese. Luteolin possesses several biological activities such as redox modulation, estrogenic or anti-estrogenic activity, anti-inflammation and anti-cancer [1]. Anti-cancer effects of luteolin are mediated by inactivating several cellular signals and transcription pathways that are important role in cancer progression such as proliferation, angiogenesis and metastasis [4]. Furthermore, luteolin is well known to have an anti-cancer property by inducing apoptosis through c-Jun activation and histone H3 acetylation-mediated Fas/FasL expressions [5], antioxidant activity [6], suppressing mitogen-activated protein kinase, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) [7] and signal transducer and activator of transcription 3 signaling pathways [8].

The balance between life and death of cells is important for maintaining tissue homeostasis. Excessive cell proliferation and too little cell death can contribute to the pathogenesis such as cancer [9,10]. Apoptosis, a type of cell death, has critical roles in the development and homeostasis of cell population. Apoptotic cells are removed by phagocytosis of phagocytes such as macrophages, leading to no inflammation [11,12]. There are two alternative pathways to induce apoptosis. One is the intrinsic pathway also known as the mitochondria pathway activated by intercellular signals, the other is the extrinsic pathway also known as the death receptor pathway activated by extracellular signals [9,13]. Caspase family proteases are key factors in both two apoptotic pathways and trigger characteristic apoptotic morphology such as cell

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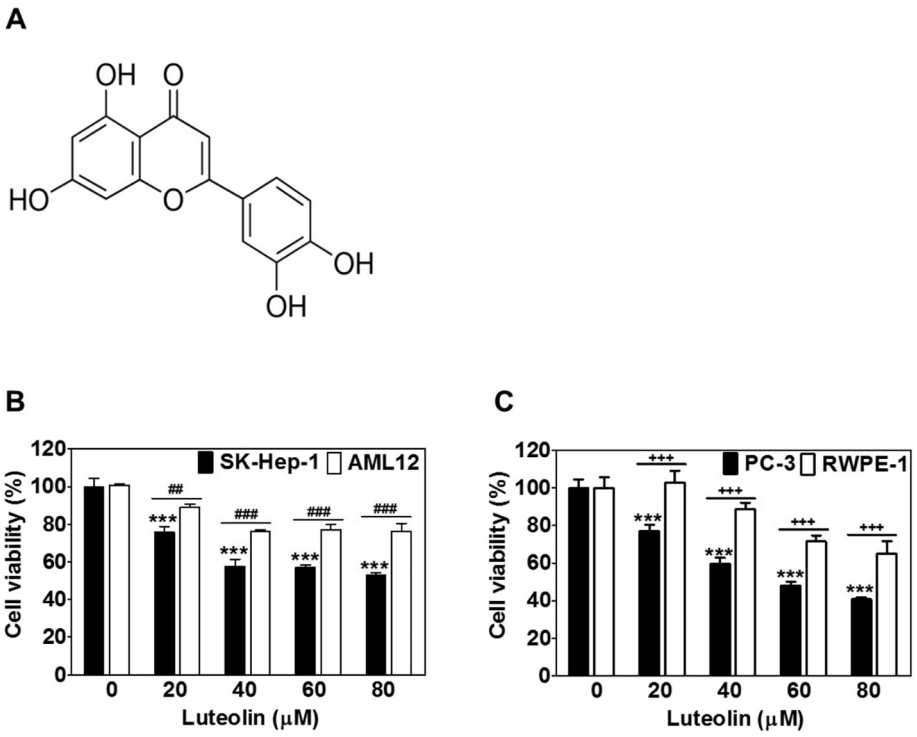

Fig. 1. Luteolin decreases the viability of SK-Hep-1 cells. (A) Chemical structure of luteolin. SK-Hep-1 cells, AML12 cells (B), PC-3 cells and RWPE-1 cells (C) were treated with various concentrations of luteolin (20, 40, 60, 80 μM) for 24 h and cell viability was determined by MTT assay. Data are expressed as mean ± SD from three independent experiments. n = 9. Results were statistically calculated by Student's *t*-test. ***$p < 0.001$ *vs.* untreated control. ##$p < 0.01$ and ###$p < 0.001$ *vs.* SK-Hep-1 cells. +++$p < 0.001$ *vs.* PC-3 cells.

Table 1
Antibodies used in this study.

Antibody	Company	Dilution	Product no.
cleaved-caspase 3	CST	1:1000	9664
cleaved-caspase 8	CST	1:1000	9496
cleaved-caspase 9	CST	1:1000	7237
cleaved-PARP	CST	1:2000	5625
XIAP	CST	1:1000	2045
Mcl-1	CST	1:1000	5453
Bid	Santa Cruz	1:1000	sc-373939
OPN	Santa Cruz	1:1000	sc-21742
β-actin	Sigma-Aldrich	1:20,000	A5316
phospho-AKT	CST	1:2000	4060
AKT	CST	1:2000	4691
goat anti-rabbit IgG-HRP	CST	1:5000	7074P2
goat anti-mouse IgG-HRP	Bio-Rad	1:5000	STAR120P

CST, Cell Signaling Technology (Beverly, MA); Santa Cruz, Santa Cruz Biotechnology (Danvers, MA); Sigma-Aldrich (St Louis, MO); Bio-Rad (Langford Lane, Kidlington).

Table 2
Primers used in this study.

mRNA	Primer sequences	Size	Annealing temperature
GAPDH	S: GCTGATGATCTTGAGGCTGTTGTC AS: ATGGGGAAGGTGAAGGTCGGAGTC	444	60
OPN	S: TTCATAACTGTCTTCCCACGGCT AS: AGTTTCGCAGACCTGACATCCAGT	161	59

shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and apoptotic body formation [14,15].

Osteopontin (OPN) is an extracellular matrix-associated, cytokine-like, noncollagenous, sialic acid rich phosphoglycoprotein [16]. OPN regulates various cancer signaling pathways by binding to its cell surface receptors integrins and CD44, leading to upregulation of several oncogenic molecules that are involved in tumor growth, angiogenesis and metastasis [16–18]. Previous studies demonstrated that OPN induced cancer cell invasion through cofilin inactivation mediated by the focal adhesion kinase/PI3K/AKT/Rho-associated kinase pathway [19–21]. Furthermore, high OPN expression is correlated with malignant transformation, pathological stage and poor outcomes such as lower overall and disease-free survival in several forms of cancer patients [22,23], suggesting that OPN may be clinically used as a prognostic and diagnostic biomarker for cancers [24,25]. Few studies reported anti-apoptotic effects of OPN on chemotherapy-induced apoptosis [26,27]. OPN knockdown using siRNA [28] and OPN-specific antibody [29] elevated apoptosis in cancer cells. Therefore, OPN is a promising target for successful treatment of cancer. However, the role of OPN in regulating apoptosis is not yet fully elucidated. Thus, this study investigated the molecular mechanism underlying the effect of luteolin on apoptosis focusing on the involvement of OPN in human hepatocellular carcinoma (HCC) SK-Hep-1 cells, which express high level of OPN.

2. Materials and methods

2.1. Cell culture

Human HCC SK-Hep-1 cells (high OPN expression) and mouse normal hepatocyte AML12 cells were obtained from Korea Cell Line Bank (KCLB, Seoul) and ATCC (Manassas, VA), respectively and maintained in DMEM with 10% fetal bovine serum (FBS) and 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C. Human prostate cancer PC-3 cells and human prostate normal RWPE-1 cells were obtained from Korea Cell Line Bank (KCLB, Seoul) and ATCC (Manassas, VA), respectively and maintained in RPMI1640 with 10% FBS and 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Cell viability assay

Cells (2×10^4) were seeded in a 96-well plate, cultured for 24 h, and treated with various concentrations (20, 40, 60 and 80 μM) of luteolin (Fig. 1A, Sigma-Aldrich, St Louis, MO) in serum-free media for 24 h. The effects of luteolin on the viability of SK-Hep-1 cells and AML12 cells were measured by

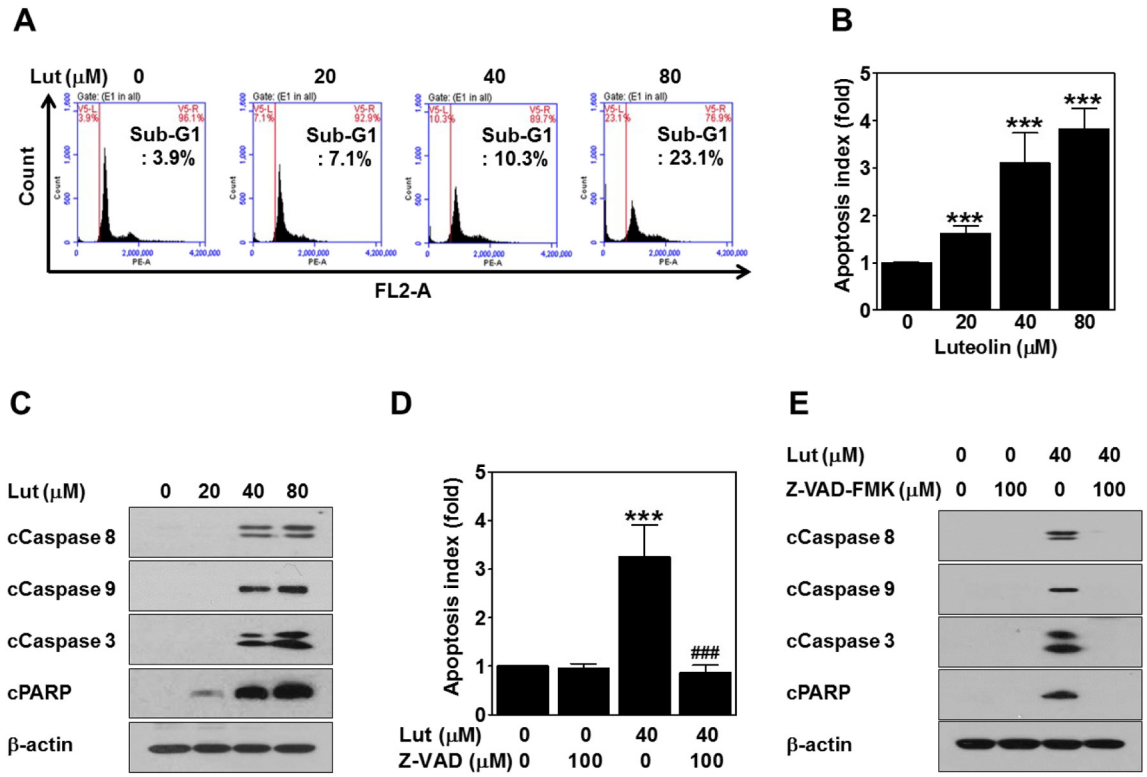

Fig. 2. Luteolin induces caspase-dependent apoptosis in SK-Hep-1 cells. Cells were treated with luteolin for 24 h. (A) Cell cycle analysis was performed after PI staining by flow cytometry system. (B) Apoptotic cell death was evaluated using a cell death detection ELISA^{PLUS} kit. n = 9. (C) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Cells were pretreated with *Z*-VAD-FMK for 1 h and then treated with luteolin for 24 h. (D) Apoptotic cell death was evaluated using a cell death detection ELISA^{PLUS} kit. n = 9. (E) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Data are expressed as mean ± SD from three independent experiments. Results were statistically calculated by Student's *t*-test. ***$p < 0.001$ *vs.* untreated control. ###$p < 0.001$ *vs.* luteolin-treated control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MO) assay as described previously [15]. MTT solution (1 mg/ml) was added for 2 h and then formazan was dissolved with DMSO (Sigma-Aldrich). The absorbance was detected by microplate reader (Sunrise RC, TECAN, Austria) at 570 nm. Cell viability was determined as a percentage of the control.

2.3. Cell cycle analysis

Cells (3.7×10^5) were seeded on a 6-well plate, treated with luteolin for 24 h and fixed in 70% ethanol at −20 °C overnight. The fixed cells were washed with PBS and incubated in 200 μl of RNase A solution (Sigma-Aldrich, 1 mg/ml) in PBS for 1 h at 37 °C. The cells were stained with 1 ml of propidium iodide solution (Sigma-Aldrich, 50 μg/ml) in PBS for 30 min at room temperature (RT). After filtering with 40 μm strainer, cell populations were analyzed by FACSCalibur flow cytometry system (Becton Dickinson, Heidelberg, Germany). The Forward scatter/Side scatter and the Pulse Width/Area were measured to identify single cells and to exclude cell doublets, respectively. For analysis, the Pulse Width/Area were gated to gate out obvious debris.

2.4. Detection of apoptotic cell death

Cells (2×10^4) were seeded in a 96-well plate and treated with luteolin for 24 h. *Z*-VAD-FMK (Adooq BioScience, Irvine, CA) or OPN (R &D system, Minneapolis, Minnesota) or SC79 (Sigma-Aldrich) was pretreated for 1 h before luteolin treatment. Apoptotic cell death was determined using a cell death detection ELISA^{PLUS} kit (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, the cells were lysed in 200 μl of lysis buffer for 30 min at RT and 20 μl of supernatant from cell lysates were transferred into a streptavidin-coated microplate. After adding 80 μl of the immunoreagent under gently shaking for 2 h at RT, the microplate was washed with PBS three times, and then 100 μl of ABTS solution were pipetted to each well for 10 min. The absorbance was detected by microplate reader (Sunrise RC, TECAN, Austria) at 405 nm after adding 100 μl of stop solution.

2.5. Western blot analysis

Cells (3.7×10^5) were seeded on a 6-well plate and treated with luteolin for 24 h. *Z*-VAD-FMK or OPN or SC79 was pretreated for 1 h before luteolin treatment. The cells were lysed in RIPA buffer containing inhibitors for phosphatases and proteases (Thermo Scientific, Rockford, IL). Total protein lysates (20 μg) were fractionated by 8, 10 or 12% SDS-PAGE gel and transferred onto a nitrocellulose transfer membrane (Pall Corporation, Port Washington, NY) for 110 min at 300 mA. The membranes were blocked with 5% nonfat skim milk or 5% bovine serum albumin for 1 h at RT and then incubated with primary antibodies at 4 °C overnight followed by specific secondary antibodies for 2 h at RT. Proteins were detected using an ECL detection kit (GE Healthcare) and quantified using ImageJ 1.40 g software (National Institute of Health, Bethesda, MD). The antibodies used in this study are listed in Table 1.

2.6. Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR)

Cells (3.7×10^5) were seeded on a 6-well plate and treated with luteolin for 24 h. Total RNA were isolated using a TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized with Oligo dT primers (Bioneer Corporation, Daejeon) and M-MLV reverse transcriptase (Promega, Madison, WI).

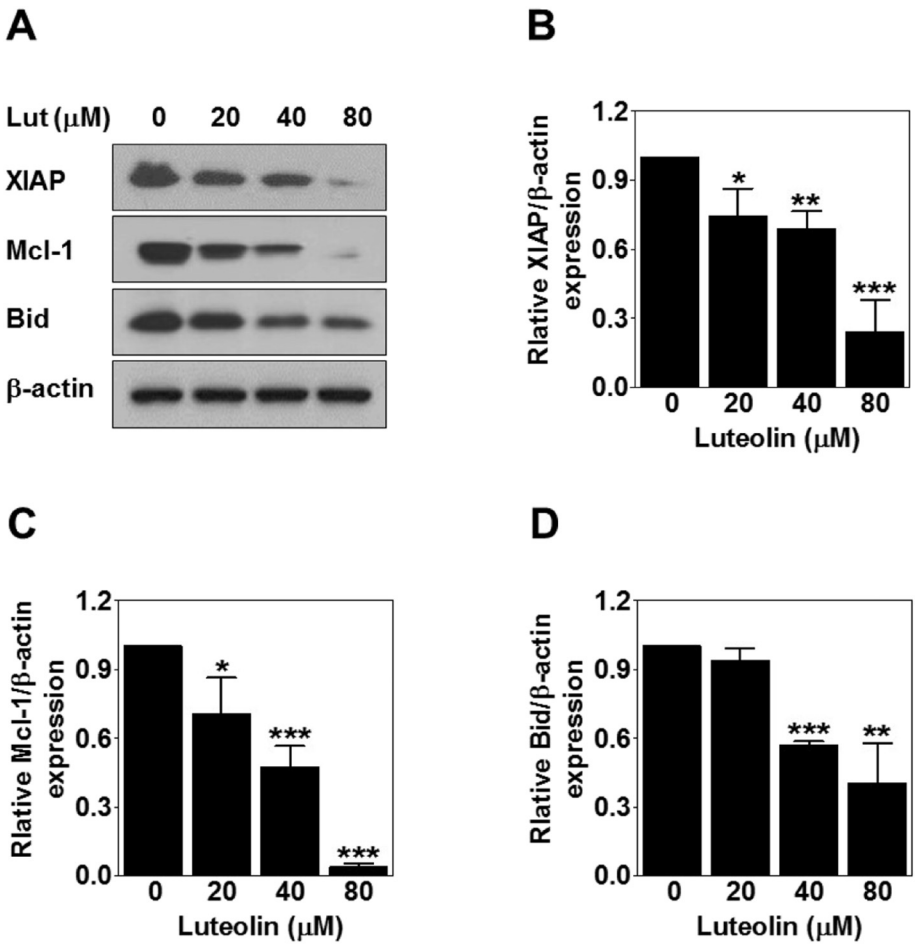

Fig. 3. Luteolin downregulates XIAP and Mcl-1, and cleaves Bid in SK-Hep-1 cells. Cells were treated with luteolin for 24 h. Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Data are expressed as mean ± SD from three independent experiments. n = 3. Results were statistically calculated by Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* untreated control.

PCR was performed using EconoTaq PLUS GREEN 2× Master mix (Lucigen, Middleton, WI) according to the manufacturer's protocol. The PCR products were detected on 2% agarose gels and quantified using ImageJ 1.40 g software (National Institute of Health). The primers used in this study are listed in Table 2.

2.7. Statistical analysis

All data are expressed as mean ± SD from three independent experiments. Cell viability assay and apoptotic cell death detection were conducted in triplicate. Results were statistically calculated by Student's *t*-test using Sigma plot software (Systat Software Inc., San Jose, CA). Values of $p < 0.05$ were considered significant differences.

3. Results

3.1. Luteolin has little toxicity to normal cells compared with cancer cells

To evaluate the cytotoxic effect of luteolin on cancer cells and normal cells, human HCC SK-Hep-1 cells and mouse normal hepatocyte AML12 cells were treated with luteolin, respectively. After 24 h incubation, MTT assay was carried out. Luteolin significantly decreased the viability of SK-Hep-1 cells and PC-3 cells in a dose-dependent manner. However, cytotoxic effect of luteolin is higher in cancer cell line than in normal cell line, which is statistically significant (Fig. 1B). To confirm this effect, MTT assay was performed in human prostate cancer PC-3 cells and human prostate normal RWPE-1 cells after luteolin treatment for 24 h. As expected, luteolin was less toxic to normal cells compared with cancer cells (Fig. 1C). These results indicated that luteolin may be a good anti-cancer agent with little toxicity.

3.2. Luteolin induces caspase-dependent apoptosis in SK-Hep-1 cells

To identify whether the cytotoxic effect of luteolin on SK-Hep-1 cells is relative to apoptosis, cell cycle analysis was performed in luteolin-treated cells for 24 h. Apoptotic cell population, sub-G_1, was increased by 7.1, 10.3 and 23.1% at 20, 40, and 80 μM of luteolin, respectively, compared with control (3.9%) (Fig. 2A). To confirm apoptotic effect of luteolin, apoptosis index was calculated under the same condition using a cell death detection ELISA kit. As shown in Fig. 2B, luteolin led to a significant increase in apoptosis in a dose-dependent manner. These results demonstrated that luteolin induces apoptosis in SK-Hep-1 cells.

To explore the role of luteolin in inducing apoptosis, caspases and PARP were detected by western blot after 24 h treatment with luteolin in SK-Hep-1 cells. Luteolin dramatically elevated cleaved-caspase 8, −9 and −3, and cleaved-PARP indicating an activation of caspases (Fig. 2C). To further investigate whether luteolin-activated caspases triggers apoptosis, cell death detection and western blot were assessed after luteolin or/and *Z*-VAD-FMK, a pan caspase inhibitor, treatment. Cell death detection results showed that apoptosis was significantly increased by 40 μM of luteolin, which was strikingly decreased by 100 μM of Z-VAD-FMK (Fig. 2D). Also, western blot results showed that luteolin activated caspase 8, −9 and −3 followed by cleavage of PARP,

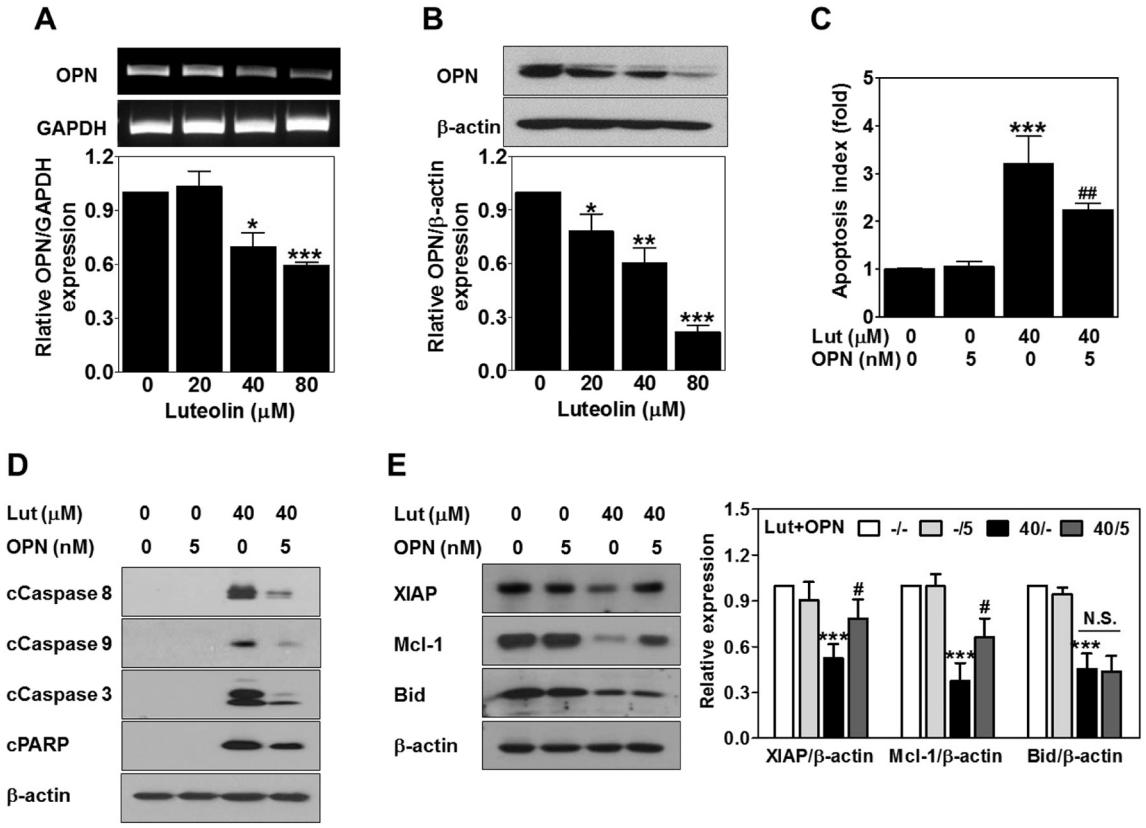

Fig. 4. Luteolin induces apoptosis by inhibiting OPN expression in SK-Hep-1 cells. Cells were treated with luteolin for 24 h. (A) OPN mRNA level was analyzed by RT-PCR. GAPDH is a loading control. n = 3. (B) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Cells were pretreated with OPN for 1 h and then treated with luteolin for 24 h. n = 3. (C) Apoptotic cell death was evaluated using a cell death detection ELISA^{PLUS} kit. n = 9. (D and E) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Data are expressed as mean ± SD from three independent experiments. n = 3. Results were statistically calculated by Student's *t*-test. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ *vs.* untreated control. $\#p < 0.05$ and $\#\#p < 0.01$ *vs.* luteolin-treated control. N.S.: not significant.

which was completely blocked by Z-VAD-FMK treatment (Fig. 2E). These results verified that luteolin induces apoptosis, which is caspase-dependent pathway in SK-Hep-1 cells.

3.3. Luteolin downregulates XIAP and Mcl-1, and cleaves Bid in SK-Hep-1 cells

To further delineate the apoptotic effect of luteolin, western blot was conducted to estimate the expression of apoptosis-related proteins such as inhibitor of apoptosis protein family (IAP, XIAP and survivin), anti-apoptotic B-cell lymphoma-2 (Bcl-2) family proteins (Mcl-1 and Bcl-2) and pro-apoptotic bcl-2 family proteins (Bid and Bax) after luteolin treatment for 24 h. There is no significant difference between control cells and luteolin-treated cells in the expression of survivin, Bcl-2 and Bax (data not shown). However, luteolin significantly downregulated the expression of XIAP and Mcl-1, and cleaved Bid in a dose-dependent manner (Fig. 3). These results demonstrated that luteolin-induced apoptosis is associated with IAP and Bcl-2 family.

3.4. Luteolin induces apoptosis by inhibiting OPN expression in SK-Hep-1 cells

Since SK-Hep-1 cells express high level of OPN, to explore whether luteolin affects OPN expression to induce apoptosis, cells were treated with luteolin for 24 h and then mRNA level and protein level for OPN were detected by RT-PCR and western blot, respectively. As shown in Fig. 4A and B, luteolin significantly downregulated OPN expression at mRNA and protein level in a dose-dependent manner. To examine the involvement of OPN in luteolin-induced apoptosis, cells were treated with 5 nM of exogenous OPN for 1 h before luteolin treatment for 24 h and then cell death detection and western blot were analyzed. Cell death detection results showed that apoptosis was significantly elevated in luteolin-treated cells, which was decreased by OPN treatment (Fig. 4C). Western blot results showed that luteolin activated caspase 8, −9 and −3 followed by cleavage of PARP, which was dramatically blocked by OPN treatment (Fig. 4D). Furthermore, luteolin downregulated the protein expression of XIAP and Mcl-1 and Bid, which was significantly rescued by OPN but not Bid (Fig. 4E). These results revealed that OPN has a protective effect against apoptosis induced by luteolin indicating that luteolin induces apoptosis by inhibiting OPN expression.

3.5. Luteolin induces apoptosis by inhibiting the AKT/OPN pathway in SK-Hep-1 cells

To investigate the effect of luteolin on the AKT pathway, cells were treated with 40 μM of luteolin for 1, 2 and 24 h and phospho-AKT and AKT were detected by western blot. Luteolin effectively decreased the AKT phosphorylation in a time-dependent manner, whereas total AKT remained unchanged (Fig. 5A). To evaluate the role of the AKT pathway in luteolin-induced apoptosis, cells were treated with 1 μg/ml of SC79, an AKT activator, for 1 h before luteolin treatment for 24 h and then cell death detection and western blot were carried out. Luteolin caused a significant decrease in OPN protein expression, which was significantly rescued after SC79 treatment (Fig. 5B). This result indicated that AKT is one of the upstream signaling molecules of OPN and luteolin downregulated OPN expression by inhibiting AKT activity. Cell death detection results showed that apoptosis was significantly induced in

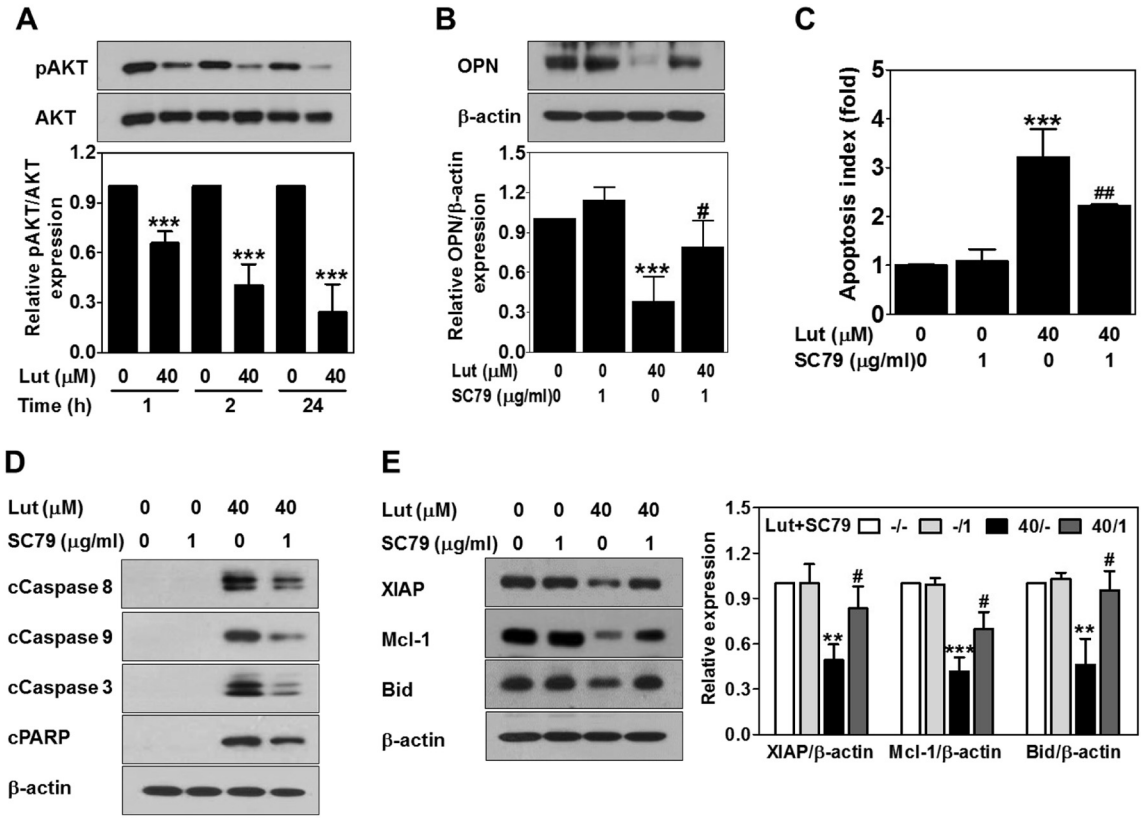

Fig. 5. Luteolin induces apoptosis by inhibiting the AKT/OPN pathway in SK-Hep-1 cells. (A) Cells were treated with luteolin for 1, 2 and 24 h. Total protein lysates analyzed by western blot using specific antibodies. Cells were pretreated with SC79 for 1 h and then treated with luteolin for 24 h. n = 3. (B) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. n = 3. (C) Apoptotic cell death was evaluated using a cell death detection ELISA^{PLUS} kit. n = 9. (D and E) Total protein lysates analyzed by western blot using specific antibodies. β-actin is a loading control. Data are expressed as mean ± SD from three independent experiments. n = 3. Results were statistically calculated by Student's *t*-test. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* untreated control. $\#p < 0.05$ and $\#\#p < 0.01$ *vs.* luteolin-treated control.

luteolin-treated cells, which was blocked by SC79 treatment (Fig. 5C). Western blot results showed that luteolin activated caspase 8, −9 and −3, and cleavaged PARP, which was decreased by SC79 treatment (Fig. 5D). Furthermore, luteolin downregulated the protein expression of XIAP and Mcl-1 and Bid, which was significantly rescued by SC79 (Fig. 5E). These results demonstrated that luteolin-induced apoptosis results from inhibiting the AKT/OPN pathway.

4. Discussion

Luteolin is a flavonoid found in fruits, vegetables and medicinal herbs [1]. Although luteolin is well-known to induce apoptosis in various types of cancers [6–8], the involvement of OPN in luteolin-induced apoptosis is not well defined. This study investigated the molecular mechanism underlying the effect of luteolin on apoptosis focusing on the role of OPN in human HCC SK-Hep-1 cells with high level of OPN.

In this study, luteolin strikingly induced apoptosis in HCC with little toxicity to normal cells. It means that the side effects of luteolin may be less. The development of compounds with low toxicity is an essential part of cancer therapies. [15,30]. Apoptosis is initiated through extrinsic and intrinsic pathways [9,13]. Both apoptotic pathways activate caspase family proteases, which are key regulators in apoptosis. Initiator caspases 8 and −9 are activated by extrinsic and intrinsic pathways, respectively, which in turn activate executioner caspase 3 followed by the cleavage of their substrates such as PARP [14,31]. In this study, luteolin activated caspase 8, −9 and −3, and cleaved PARP. *Z*-VAD-FMK treatment indicated that caspase activation is a crucial step in luteolin-induced apoptosis. These results indicated that luteolin-induced apoptosis is involved in both extrinsic and intrinsic apoptotic pathways, which is caspase-dependent.

Members of IAP such as XIAP and survivin block the activation of caspase 9 and −3, thereby inhibiting apoptosis [32,33]. Patients with high expression of either XIAP or survivin showed resistance to chemotherapy and shorter median survival time compared to patients with normal expression of either these [34]. Suppressing XIAP and survivin expression can sensitize cancer cells to apoptosis [35,36] Previous study reported that luteolin reduces survivin expression [37], whereas this study observed that luteolin downregulates only XIAP expression but not survivin (data not shown).

Bcl-2 family proteins have either pro-apoptotic family members such as Bax, Bak, Bim and Bid or anti-apoptotic family members such as Bcl-2, Mcl-1 and Bcl-XL. These regulate apoptosis through the intrinsic pathway involving in caspases 9 and −3 activation [38,39]. Previous study showed that luteolin upregulates Bax expression and downregulates Bcl-2 expression [6,7,37] to induce apoptosis, whereas this study showed no significant effect on these proteins in luteolin-treated cells compared with non-treated cells (data not shown). Luteolin effectively inhibited Mcl-1 expression and cleaved Bid. Bid is cleaved by active caspase 8 from extrinsic pathway and translocates to the mitochondria, which in turn promotes intrinsic pathway [38,40]. Taken together, luteolin communicates two apoptotic pathways through Bid.

OPN is a secreted glycophosphoprotein overexpressed in several tumor types [41] and plays vital roles in cancer progression such as tumor growth, survival, metastasis and angiogenesis [16–18]. OPN is correlated with inducing cell proliferation and inhibiting apoptosis [28]. OPN antagonized imatinib-induced apoptosis through the upregulation of Mcl-1 mediated by β-catenin [26]. This study revealed that luteolin downregulated OPN mRNA and protein levels. Luteolin-

induced apoptosis process including activation of caspases and downregulation of XIAP and Mcl-1 is blocked by OPN treatment, indicating anti-apoptotic effects of OPN. These results demonstrated that downregulation of OPN expression is required for inducing apoptosis by luteolin.

The AKT pathway is a signaling transduction pathway that regulates multiple cellular processes such as cell survival, proliferation, apoptosis, invasion, metastasis and angiogenesis [42–44]. This pathway promotes cell survival through upregulation of Bcl-xL and Bcl-2, and blocks apoptosis through caspase 9 phosphorylation, preventing a caspase cascade and Mdm2 phosphorylation, suppressing p53 activity [43,45]. Active AKT upregulates OPN expression in RNA and protein levels [46]. In this study, downregulation of OPN expression by luteolin is due to impairing the AKT activity. Luteolin-inhibited OPN expression was elevated by activating the AKT pathway by SC79 treatment. Also, the AKT pathway blocked luteolin-induced apoptosis process including activation of caspases and downregulation of XIAP and Mcl-1, and cleavage of Bid, indicating anti-apoptotic effects of the AKT pathway. These results identified that luteolin induced-apoptosis is mediated by inhibiting the AKT/OPN pathway.

5. Conclusion

This study verified that luteolin induces caspase-dependent apoptosis in human HCC SK-Hep-1 cells, which is triggered by both extrinsic and intrinsic pathways. However, luteolin shows slight cytotoxicity to normal cells. Luteolin downregulates the apoptosis-related protein expression of XIAP and Mcl-1, and cleaves Bid. The AKT/OPN pathway is closely associated with not only the expression of apoptosis-related proteins but also the activation of caspases. Luteolin-induced apoptosis results from inhibiting the AKT/OPN pathway. These results provide new insight into the involvement of OPN in luteolin-induced apoptosis and suggest that luteolin may be an attractive therapeutic strategy to treat cancers that express high level of OPN with little toxicity.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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